



THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

# Lactulose drives a reversible reduction and qualitative modulation of the faecal microbiota diversity in healthy dogs

### Citation for published version:

Ferreira, M, Salavati Schmitz, S, Schoenebeck, J, Clements, D, Campbell, S, Gaylor, D, Mellanby, R, Gow, A & Salavati, M 2019, 'Lactulose drives a reversible reduction and qualitative modulation of the faecal microbiota diversity in healthy dogs', *Scientific Reports*, vol. 9, 13350. <https://doi.org/10.1038/s41598-019-50090-7>

### Digital Object Identifier (DOI):

[10.1038/s41598-019-50090-7](https://doi.org/10.1038/s41598-019-50090-7)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Publisher's PDF, also known as Version of record

### Published In:

Scientific Reports

### Publisher Rights Statement:

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.  
© The Author(s) 2019

### General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

### Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



OPEN

# Lactulose drives a reversible reduction and qualitative modulation of the faecal microbiota diversity in healthy dogs

Marisa da Fonseca Ferreira , Silke Salavati Schmitz, Jeffrey Joseph Schoenebeck , Dylan Neil Clements, Susan Mary Campbell, Donna Elaine Gaylor, Richard J. Mellanby, Adam George Gow & Mazdak Salavati 

Hepatic encephalopathy is a frequent and debilitating complication of liver disorders. Lactulose is an established and reasonably effective treatment, yet with incompletely understood mechanisms of action. The aims of this study were to examine how the faecal microbiota composition changed before, during and after lactulose treatment in a large animal model. Healthy, privately owned dogs ( $n = 18$ ) completed a prospective cohort study. Faecal samples were collected weekly, while the subjects were either on their usual diet (week 1), or a standardised diet (weeks 2–9), with added oral lactulose in weeks 6–7. DNA extraction and 16S rRNA gene sequencing were undertaken. Faecal samples from week 7 had a significantly lower microbiota richness/diversity, based on observed operational taxonomic units, Shannon/Chao1 indexes and Pielou's Evenness. Beta diversity based on UniFrac distances was significantly different in week 7 compared to weeks 1, 5 and 9. At the phylum level, week 7 was associated with a significant increase of Firmicutes and Actinobacteria, and a decrease of Bacteroidetes and Fusobacteria, when compared to weeks 5 and 9. In summary, we have shown that lactulose induces a reversible qualitative and quantitative change of the faecal microbiota, which may explain its clinical efficacy in the management of hepatic encephalopathy.

Hepatic encephalopathy (HE) is a frequent and debilitating neurological complication in patients with liver disease. Severe (grades 3–4) HE is associated with higher in-hospital and 30-day mortality, independently of extra-hepatic organ failures<sup>1</sup>, and higher liver transplantation 90-day wait list mortality<sup>2</sup>. Covert (minimal and grade 1) HE directly results in human morbidity, being an independent predictor of reduced health-related quality of life and poor sleep quality<sup>3</sup>. Furthermore, HE contributes to a substantial economic burden. In the USA alone, total HE-related hospitalisation charges amounted to \$7.245 billion in 2009<sup>4</sup>, and up to \$58,625 per patient in 2012<sup>5</sup>.

The pathogenesis of HE is not fully understood<sup>6</sup>. Several neurotoxins and precipitating factors have been implicated, with ammonia being the most well characterised one. In advanced liver disease, this gut-derived neurotoxin may accumulate in the blood and in the brain, due to lack of hepatic conversion into urea and subsequent urinary excretion<sup>7</sup>. Clinically, in human cirrhosis, plasma ammonia has been correlated with both the severity of HE and the frequency of other organ failures, and was identified as an independent predictor of 28-day mortality<sup>8</sup>.

Lactulose, a synthetic non-absorbable disaccharide, is a commonly used medication, with or without the addition of the antibiotic rifaximin, for both the treatment and prevention of HE, with a reasonable evidence of efficacy and added benefits in reducing morbidity and mortality<sup>9,10</sup>. The postulated benefits of lactulose include: (1) decreased colonic transit time and pH, leading to decreased ammonia production and absorption; (2) increased bacterial assimilation of ammonia; (3) decreased bacterial generation of ammonia; (4) production shift from toxic to non-toxic short-chain fatty acids (SCFA); and (5) reduced bacterial DNA translocation<sup>11,12</sup>. Yet, its mechanisms of action remain incompletely elucidated.

The Royal (Dick) School of Veterinary Studies and The Roslin Institute, The University of Edinburgh, Easter Bush Campus, Roslin, Midlothian, EH25 9RG, United Kingdom. Adam George Gow and Mazdak Salavati jointly supervised this work. Correspondence and requests for materials should be addressed to M.d.F.F. (email: [marisa.ferreira@ed.ac.uk](mailto:marisa.ferreira@ed.ac.uk))

Received: 15 November 2018

Accepted: 3 September 2019

Published online: 16 September 2019

Usual diet	Standardised diet				Standardised diet + oral lactulose		Standardised diet	
Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9

**Figure 1.** Longitudinal interventions in cohort of healthy owned dogs, designed to assess the faecal microbiota.

Faecal dysbiosis is known to occur in covert and overt HE. Cirrhotic patients with minimal HE (MHE) harbour a higher proportion of urease-producing *Streptococcus salivarius* in stool samples, positively correlating with serum ammonia accumulation<sup>13</sup>. Additionally, the cirrhosis dysbiosis ratio (CDR), a previously validated ratio of autochthonous to non-autochthonous taxa in stool samples of cirrhotic patients, is lowered after development of severe HE, indicating worsened dysbiosis, and associated with 30-day mortality and organ failure<sup>14</sup>. Moreover, the presence of specific bacterial families (*Alcaligenaceae*, *Porphyromonadaceae*, *Enterobacteriaceae*) is strongly associated with poor cognition and inflammation in HE patients<sup>15</sup>. Considering the ongoing evidence regarding microbiome disruption in cirrhosis and HE<sup>16</sup>, it is likely that manipulation of the microbiota may contribute to improved outcomes. Interventions with proposed positive impact have so far included probiotics<sup>17</sup>, diet<sup>18</sup> and faecal microbiota transplantation<sup>19</sup>.

The impact of lactulose in ameliorating cirrhosis and HE-associated faecal dysbiosis is controversial. A direct impact has been supported by studies based on culture-dependent methodologies, namely: increased *Bifidobacterium*, *Lactobacillus* and *Bacteroidaceae* colonies, and reduced *Enterobacteriaceae*, *Enterococcus* and yeasts in patients with MHE, alongside with improved blood ammonia, psychometric tests and reduced risk of developing overt HE<sup>20</sup>; and increased total aerobic and anaerobic bacterial counts, and lactobacilli in cirrhotic patients without clinical HE, alongside with decreased faecal pH<sup>21</sup>. Conversely, studies based on culture-independent techniques have not substantiated an effect of lactulose in the microbiome of cirrhotic patients without HE<sup>22</sup> and have reported only a minimal change in cirrhotic patients with HE<sup>14</sup>, including after lactulose withdrawal<sup>23</sup>.

However, no study using next generation sequencing techniques has assessed quantitative and qualitative changes of the intestinal or faecal microbiome, i.e. changes based on both the abundance and the presence or absence of microbial communities, after lactulose treatment in patients entirely naïve to lactulose. The effects of oral lactulose on the faecal microbiome of healthy humans have only been evaluated through either culture-based<sup>24–26</sup> or culture-independent methods targeting predominant bacterial groups<sup>27–30</sup>. As diet was only standardised in two of those studies<sup>24,30</sup>, it seems likely that a variable diet could have impacted results<sup>31</sup>.

The human faecal microbiome is closer to the canine faecal microbiome when compared to pigs or mice<sup>32</sup>. Dogs evolved to cohabit with people, and hence adapted to a similar diet<sup>33</sup>. As they are frequently kept as companion animals, they are also exposed to the same environment. In addition, dogs can equally suffer from HE and lactulose is frequently used as supportive treatment in this condition<sup>34</sup>. Consequently, companion dogs can represent a useful comparative model to explore the faecal microbiome in HE as well as the effects of certain interventions on its composition, richness and function.

Therefore, the aims of this study were to investigate the magnitude and duration of quantitative and qualitative changes of the faecal microbiota by lactulose in healthy privately-owned dogs fed a standardised commercial diet. It was hypothesised that oral lactulose administration would significantly and transiently change the faecal microbiota in healthy dogs.

## Methods

**Prospective cohort study design.** Dogs owned by members of staff at the Hospital for Small Animals, the Royal (Dick) School of Veterinary Studies, University of Edinburgh, were recruited with the following inclusion criteria: no current history of any disease; up to date vaccination and deworming records; and no current or recent administration of medications. A faecal sample was requested to be collected from each subject weekly, pertaining to the interventions schematised in Fig. 1.

The standardised diet was a commercial maintenance diet for adult dogs (Hill's™ Science Plan™ Canine Adult Advanced Fitness™ Large Breed with Chicken, Hill's Pet Nutrition Ltd., Guildford, UK) and the lactulose was a 3.5 g/5 ml oral solution (Sandoz Ltd, Hampshire, UK). The dose of lactulose was calculated at 0.5 ml/kg and given every 12 hours, unless excessively soft or unformed faeces were noticed, at which point the subjects' owners would notify one of the authors (MFF) and subsequently decrease the dose by 25% each time, aiming to achieve a soft faecal consistency that would be still amenable for manual collection.

Faecal samples were collected into plain bijoux tubes, kept frozen at  $-20^{\circ}\text{C}$  for a maximum of 24 hours and transferred afterwards to a  $-80^{\circ}\text{C}$  archiving freezer.

Informed consent was obtained from each subject's owner. The study was approved by the University of Edinburgh's Veterinary Ethical Review Committee (reference number 112–14) and carried out in accordance with the institution's relevant guidelines and regulations.

**Faecal DNA extraction, amplification and sequencing.** Each sample was defrosted, manually homogenised and DNA extraction performed with a commercial kit (PowerSoil® DNA Isolation Kit, MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions<sup>35</sup>. Amplification of DNA was undertaken with PCR of the hypervariable V4 region of the 16S ribosomal RNA (rRNA) gene, using dual-indexing primers (515F/806R), followed by amplicon quantification (Quant-iT™ PicoGreen®, Invitrogen,

Ltd., Paisley, UK) and pooling, as previously described<sup>36,37</sup>. Standard 16S rRNA library preparation and sequencing with the Illumina® MiSeq® (v2 150PE) platform were performed by Edinburgh Genomics (The University of Edinburgh, UK).

**Data analysis.** Software packages for data analysis included the Quantitative Insights Into Microbial Ecology (QIIME2™, <https://qiime2.org/>) pipeline, RStudio® (version 1.1.453, © 2009–2018 RStudio, Inc., Boston, MA, US) with the package qiime2R (v0.12), as well as GraphPad Prism® (version 7, GraphPad Software Inc., La Jolla, CA, US).

The paired-end raw reads (Supplementary Data Mendeley Data) were analysed using QIIME2™. Briefly, sequence data with sequence quality information was imported through the “fastq manifest” format *PairedEndFastqManifestPhred33*. Demultiplexed sequence counts were summarised showing a median of 58,478 sequences per sample, with a minimum of 27,297 and maximum of 134,220. Sequence quality trimming, chimera filtering and feature table construction was performed through the DADA2 pipeline<sup>38</sup>. Mapping of feature identifiers to sequences was created using links for the Basic Local Alignment Search Tool (BLAST)<sup>39</sup>, and multiple sequence alignment of the sequences was undertaken by the Fast Fourier Transform (MAFFT) program<sup>40</sup>. This was followed by filtering of the alignment with the mask plugin<sup>41</sup>, generation of a phylogenetic tree with the FastTree program<sup>42</sup> and application of midpoint rooting. The QIIME2™ *q2-diversity* plugin was used for rarefaction analysis and computation of alpha diversity metrics (observed operational taxonomic units [OTUs], Shannon diversity index, Chao1 and Pielou’s Evenness), as well as beta diversity metrics (weighted and unweighted UniFrac distances). Finally, assigning taxonomy to sequences was performed with a pre-trained Naïve Bayes classifier (Greengenes 13.8 99% OTUs) through the QIIME2™ *q2-feature-classifier* plugin<sup>43</sup>.

A human-extrapolated CDR: ratio of commensal autochthonous taxa (*Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae*, and *Clostridiales* Insertae Sedis XIV) to potentially pathogenic non-autochthonous taxa (*Enterobacteriaceae* and *Bacteroidaceae*)<sup>44</sup>, was calculated without the inclusion of *Clostridiales* Insertae Sedis XIV, as this taxon was not detected in the studied population.

Statistical analyses used included descriptive statistics, as well as the following inferential statistical tests: Kruskal–Wallis test to compare differences between subjects regarding alpha diversity metrics; Wilcoxon matched-pairs signed rank test to compare differences between weeks regarding alpha diversity metrics, taxonomy frequencies and the CDR; paired t-test to compare differences between weeks regarding the presence of *Lachnospiraceae*; and Permutational Multivariate Analysis of Variance (PERMANOVA) test to compare differences between subjects and weeks regarding beta diversity metrics<sup>45</sup>. Normality was assessed with the Shapiro–Wilk test. Statistical significance level was set at  $p < 0.05$ .

## Results

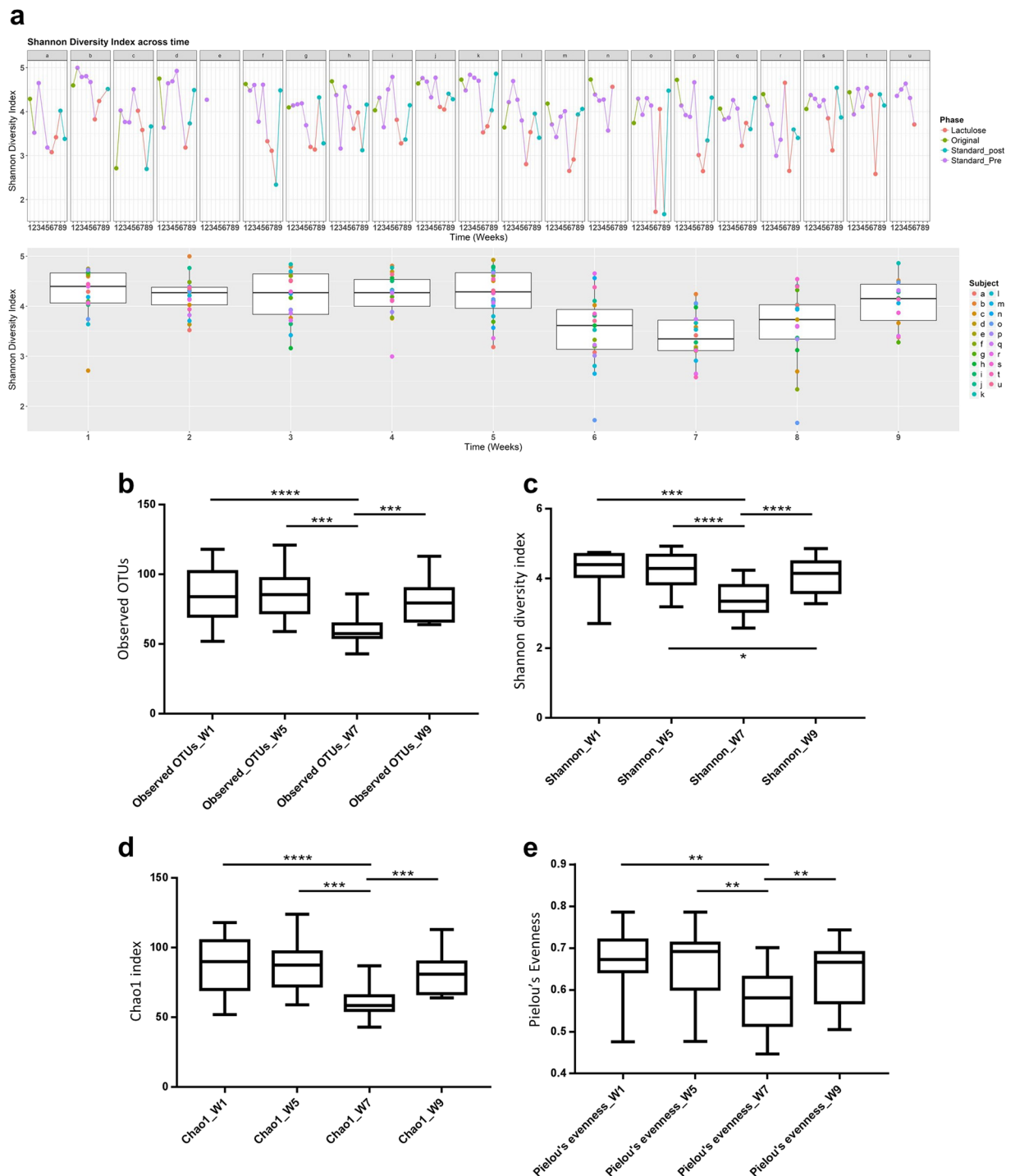
**Population’s baseline characteristics.** A total of 21 dogs were enrolled, with a median age of 5 years (range of 2–10 years). Sex distribution included 12 females (11 neutered) and 9 males (7 neutered). Just over half ( $n = 11$ ) of the cohort was represented by crossbred dogs, with the remaining 10 dogs distributed as follows: two each of English Cocker Spaniel, English Springer Spaniel, Border Collie and Podenco; and one each of Boston Terrier and Labrador Retriever.

**Adherence to study protocol and side effects related to lactulose administration.** Three dogs did not complete the study for the following reasons: acute diarrhoea following dietary indiscretion ( $n = 1$ ); requirement of a NSAID (meloxicam) for pain management secondary to presumptive degenerative joint disease ( $n = 1$ ); and lip fold dermatitis after being licked by another dog in the household once starting lactulose ( $n = 1$ ). In addition, two faecal samples (one each from weeks 4 and 8) were accidentally not collected. In total, 172 faecal samples were analysed, one of which failed sequencing (week 6). Side effects associated with lactulose administration included excessively soft faeces ( $n = 7$ ) and unformed faeces ( $n = 1$ ), all resolving after a dose reduction of 25% and 50%, respectively, therefore not requiring a drop out from the study.

**Alpha diversity.** Community richness and diversity values were different between subjects regarding the following metrics: observed OTUs ( $p < 0.0001$ ), Shannon diversity index ( $p = 0.0025$ ), Chao1 index ( $p < 0.0001$ ) and Pielou’s Evenness ( $p < 0.0001$ ). Assessment of community richness and diversity across time is shown in Fig. 2. There was a reduction of all the above metrics at week 7 (standardised diet and oral lactulose), when compared to weeks 1 (usual diet), 5 (standardised diet) and 9 (standardised diet after having stopped lactulose). Conversely, values from weeks 1, 5 and 9 didn’t differ from each other apart from Shannon diversity index values between week 5 and 9 (Table 1).

**Beta diversity.** Bacterial community presence/absence (qualitative) and abundance (quantitative) assessments with unweighted and weighted UniFrac distances, respectively, are illustrated in Fig. 3. These were overall different between subjects ( $p < 0.001$ ). When analysed across time, the distances at week 7 were different from weeks 1, 5 and 9. However, values at weeks 1, 5 and 9 didn’t differ from each other (Table 2).

**Taxonomy.** The relative and absolute distribution of phyla abundance across time is depicted in Fig. 4. Irrespective of week number (5, 7 or 9), the most abundant phylum was Firmicutes, followed by Bacteroidetes. A shift of phyla was observed at week 7, with the third most abundant phylum being Actinobacteria, followed by Fusobacteria and Proteobacteria. In contrast, for both weeks 5 and 9, Fusobacteria was the third most abundant phylum, followed by Proteobacteria and Actinobacteria. Moreover, when compared to weeks 5 and 9, week 7 was associated with a higher abundance of both Firmicutes ( $p = 0.0056$  and  $0.0047$ , respectively) and Actinobacteria ( $p = 0.0090$  and  $0.0104$ , respectively), and lower abundance of both Bacteroidetes ( $p = 0.0304$  and  $0.0120$ , respectively) and Fusobacteria ( $p = 0.0040$  and  $0.0008$ , respectively). The abundance of these phyla was similar between



**Figure 2.** Variation of the canine faecal microbiota, assessed by alpha diversity metrics. **(a)** Shannon diversity index across a cohort of healthy owned dogs (letters a-u) while either on their usual diet (Original, week 1), a standard diet (Standard\_Pre, weeks 2–5), a standard diet and oral lactulose (Lactulose, weeks 6–7), or a standard diet after having stopped lactulose (Standard\_Post, weeks 8–9). **(b–e)** Box and whiskers plots (median, 25<sup>th</sup> and 75<sup>th</sup> quartiles), depicting different alpha diversity metrics at selected weeks: observed operating taxonomic units (OTUs) **(b)**; Shannon diversity index **(c)**; Chao1 index **(d)**; and Pielou's Evenness. **(e)** \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$  (Wilcoxon matched-pairs signed rank test).

weeks 5 and 9 (Firmicutes,  $p = 0.6397$ ; Actinobacteria,  $p = 0.6095$ ; Bacteroidetes,  $p = 0.8650$ ; and Fusobacteria,  $p = 0.1964$ ).

A total of 20 families were found to be present in the microbiota of at least half of the subjects at one or more time points (Fig. 5a). Different abundances across time were found in eight families (Fig. 5b), six of which showed a different abundance in week 7 when compared to weeks 5 and 9 (Fig. 5c), while both these time points

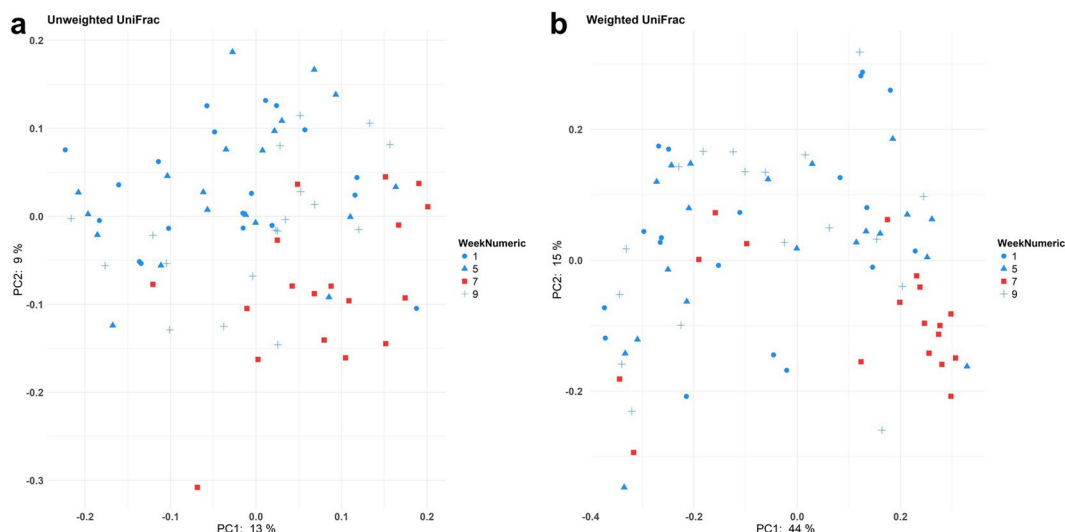


Metric	Weeks 1 vs 5	Weeks 1 vs 7	Weeks 1 vs 9	Weeks 5 vs 7	Weeks 5 vs 9	Weeks 7 vs 9
Observed OTUs <sup>a</sup>	0.8056	<0.0001	0.7904	0.0002	0.3516	0.0003
Shannon diversity index	0.9843	0.0003	0.0814	<0.0001	0.0268	<0.0001
Chao1 index	0.7453	<0.0001	0.7379	0.0002	0.2690	0.0003
Pielou's Evenness	0.7680	0.004	0.0898	0.0010	0.0898	0.009

**Table 1.** Statistical significances ( $p$ ) of alpha diversity metrics for the canine faecal microbiota when compared across time. Cohort of healthy owned dogs while either on their usual diet (week 1), a standard diet (week 5), a standard diet and oral lactulose (week 7), or a standard diet after having stopped lactulose (week 9). Statistical test used: Wilcoxon matched-pairs signed rank test. <sup>a</sup>Operational Taxonomic Units.

Metric	Weeks 1 vs 5	Weeks 1 vs 7	Weeks 1 vs 9	Weeks 5 vs 7	Weeks 5 vs 9	Weeks 7 vs 9
Unweighted UniFrac	0.534	0.001	0.136	0.001	0.272	0.012
Weighted UniFrac	0.474	0.001	0.978	0.002	0.645	0.001

**Table 2.** Statistical significances ( $p$ ) of beta diversity metrics for the canine faecal microbiota when compared across time. Cohort of healthy owned dogs while either on their usual diet (week 1), a standard diet (week 5), a standard diet and oral lactulose (week 7), or a standard diet after having stopped lactulose (week 9). Statistical test used: PERMANOVA.



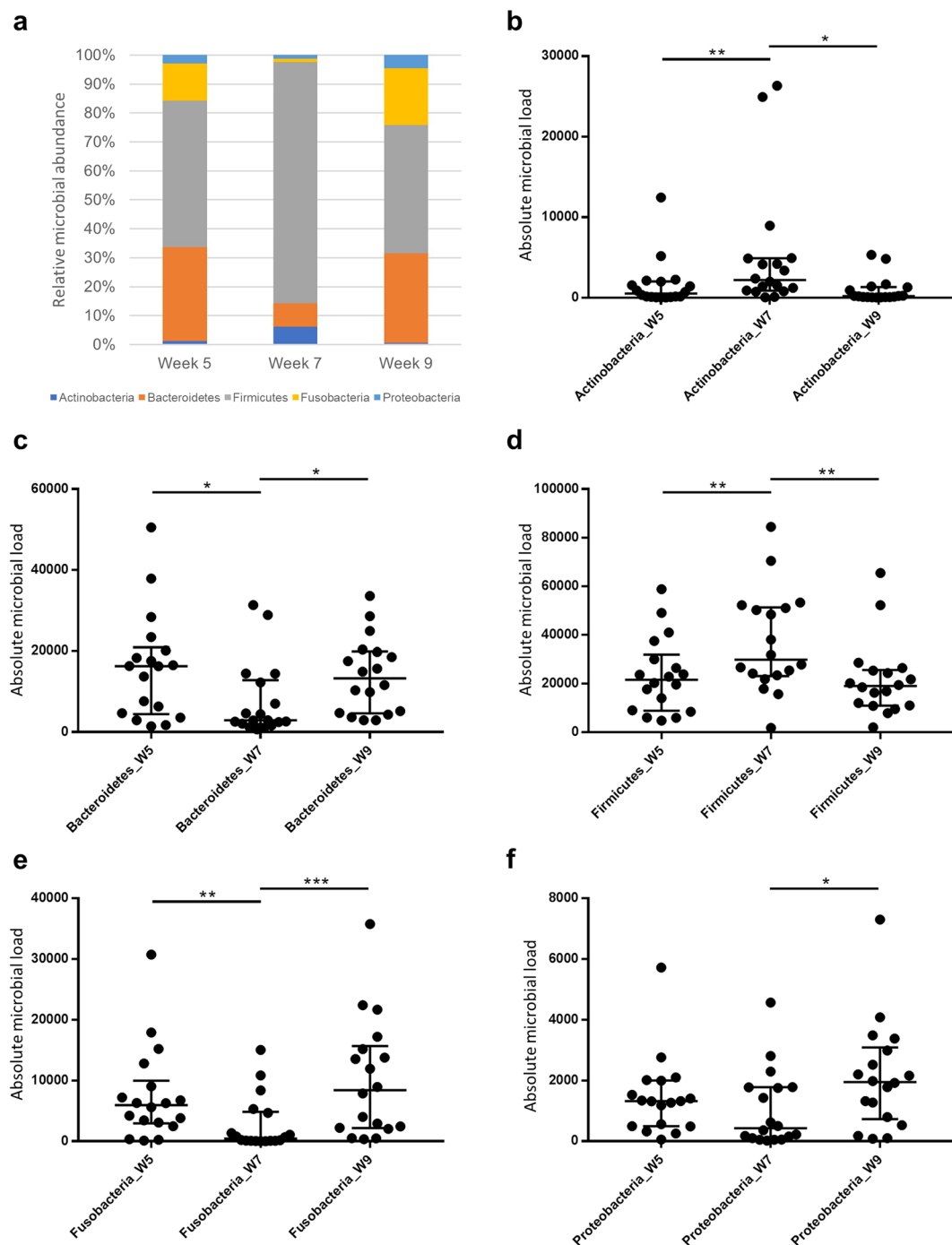
**Figure 3.** Variation of the canine faecal microbiota, assessed by beta diversity metrics. (a,b) Principal coordinate analysis based on unweighted (a) and weighted (b) UniFrac distances. Cohort of healthy owned dogs while either on their usual diet (week 1), a standard diet (week 5), a standard diet and oral lactulose (week 7), or a standard diet after having stopped lactulose (week 9). Samples from week 7 were significantly different ( $p < 0.01$ , PERMANOVA test) from weeks 1, 5 and 9, while none of the latter differed from each other.

had similar abundances. Significant changes at week 7 included an increased representation of *Veillonellaceae* and *Bifidobacteriaceae*, and a decreased abundance of *Fusobacteriaceae*, *Bacteroidaceae*, *Ruminococcaceae*, *Alcaligenaceae*, *Lachnospiraceae* and *Peptococcaceae*.

**Cirrhosis dysbiosis ratio.** When analysing the CDR across time (Fig. 6), the highest values were obtained at week 7 with a median of 25.07 (range of 0.08–460.04). These were different from week 1 (median 3.19, range 0.18–30.98,  $p = 0.0079$ ), week 5 (median 2.54, range 0.18–29.78,  $p = 0.0003$ ) and week 9 (median 2.10, range 0.24–15.75,  $p < 0.0001$ ). Conversely, the CDRs calculated for weeks 1, 5 and 9 were similar to each other ( $p = 0.5678$  for week 1 vs 5;  $p = 0.2288$  for week 1 vs 9; and  $p = 0.5509$  for week 5 vs 9).

## Discussion

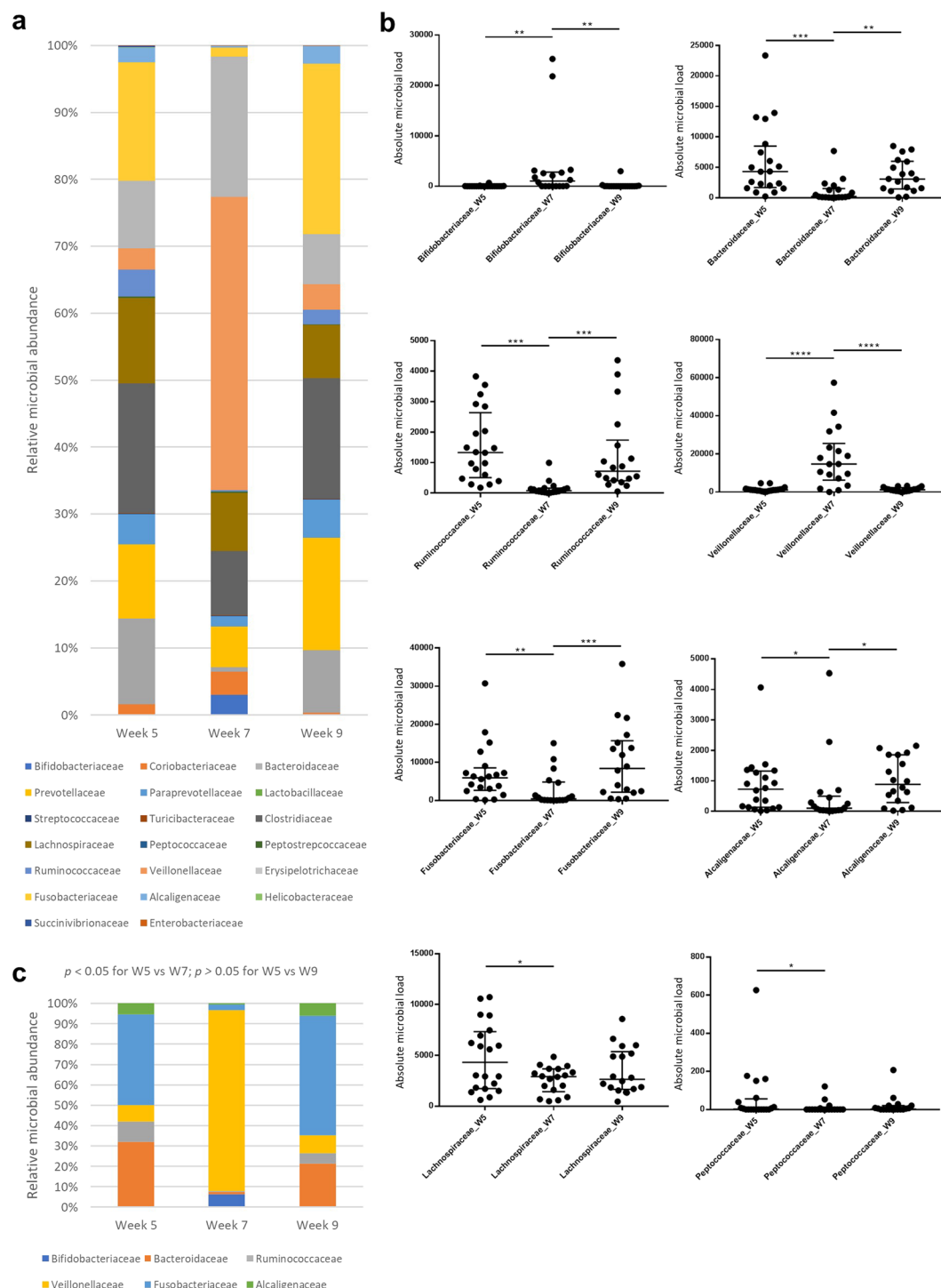
To date, there are no publications employing untargeted or global culture-independent methodologies to assess the faecal microbiome in healthy people receiving lactulose. In people with HE, further limitations often apply, given the common use of standard of care treatment by the time of study enrolment (lactulose, rifaximin, antacids), precluding an evaluation of the microbiome in non-treated HE<sup>14,18</sup>.



**Figure 4.** Abundance of phyla in the canine faecal microbiota. Cohort of healthy owned dogs while either on a standard diet (week 5), a standard diet and oral lactulose (week 7), or a standard diet after having stopped lactulose (week 9). **(a)** Median relative abundance of different phyla. **(b–f)** Dot plots (including bars for median, 25<sup>th</sup> and 75<sup>th</sup> quartiles) depicting absolute abundance of: Actinobacteria **(b)**; Bacteroidetes **(c)**; Firmicutes **(d)**; Fusobacteria **(e)**; and Proteobacteria **(f)**. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  (Wilcoxon matched-pairs signed rank test).

To the authors' knowledge, this study is the first to investigate the effect of lactulose on the canine microbiota. Collectively, lactulose induced a reversible reduction and qualitative modulation of the faecal microbiota diversity in this population of healthy dogs, while on a commercial standardised diet. Both alpha and beta diversity metrics were affected and specific taxa were implicated in this change.

The impact of lactulose has been studied in healthy mice and pigs through culture-independent methods, most showing an increased alpha diversity, which is in contrast to the present study<sup>46–49</sup>. However, experimental animals are kept in laboratory-controlled conditions and both species are known to have a more distant

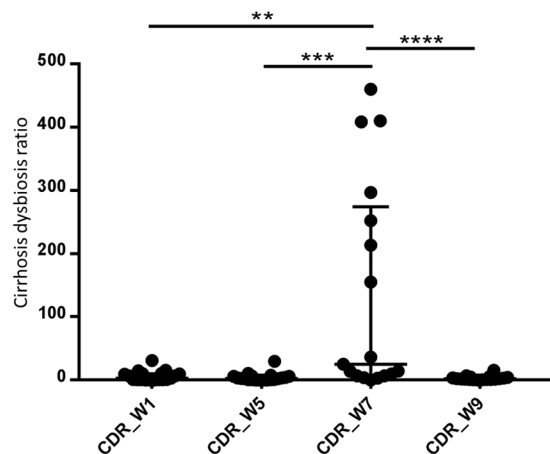


**Figure 5.** Abundance of families in the canine faecal microbiota. Cohort of healthy owned dogs while either on a standard diet (week 5), a standard diet and oral lactulose (week 7), or a standard diet after having stopped lactulose (week 9). **(a)** Median relative abundance of families with counts present in at least 50% of dogs in at least one week group. **(b)** Dot plots (including bars for median, 25<sup>th</sup> and 75<sup>th</sup> quartiles) depicting absolute abundance of statistically significant families (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; Wilcoxon matched-pairs signed rank test). **(c)** Median relative abundance of statistically significant families (Wilcoxon matched-pairs signed rank test).

microbiome from humans relative to dogs<sup>32</sup>. Assessing companion dogs, alongside maintaining the advantage of dietary control, may be therefore valuable given their natural shared environment with humans<sup>31</sup>.

No significant changes of the microbiota were observed in this study due to diet change alone. This is likely explained by the relative similarity of composition of commercial diets (most of the original diets consisted of





**Figure 6.** Cirrhosis dysbiosis ratio (CDR), calculated from the canine faecal microbiota, extrapolated from humans. Cohort of healthy owned dogs while either on their usual diet (week 1), a standard diet (week 5), a standard diet and oral lactulose (week 7), or a standard diet after having stopped lactulose (week 9). \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$  (Wilcoxon matched-pairs signed rank test).

various brands of dry dog food), in comparison to homemade or raw food<sup>50,51</sup>. There were marked inter-individual differences concerning overall alpha and beta diversity, which was not surprising, given the non-relatedness of the subjects and the diversity of breeds evaluated<sup>52,53</sup>.

Significant changes in the abundance of main phyla were observed with the introduction of lactulose and matched by changes noted at family taxa. Within the Firmicutes and Actinobacteria, *Veillonellaceae* and *Bifidobacteriaceae* increased; and decreases in *Bacteroidaceae* and *Fusobacteriaceae* likely reflect lower abundances of Bacteroidetes and Fusobacteria.

*Veillonellaceae* was the most abundant family after the administration of lactulose. Its significance in liver disease has been controversial, as previous studies have reported both increases and decreases in human cirrhosis with and without HE<sup>13,14</sup>. Bacteria of this family convert lactate to acetate and butyrate<sup>54</sup>. Previously, the latter has been positively correlated with the presence of *Veillonellaceae* in canine faeces<sup>55</sup>. Increases in microorganisms producing these SCFA could be beneficial, as in people, acetate was negatively correlated with pro-inflammatory cytokines in cirrhosis, and butyrate was protective against the development of HE<sup>16</sup>. The administration of lactulose has been associated with increased acetate and butyrate production, together with decreased branch-chained fatty acids isobutyrate and isovalerate<sup>46,47,56</sup>. Similarly to the present study, several others have reported increased numbers of faecal *Bifidobacteriaceae*<sup>24–30</sup>, which produce lactate and acetate<sup>57</sup>, as well as contribute to metabolic cross-feeding, stimulating other butyrate-producing bacteria<sup>58</sup>.

Reductions of both *Fusobacteriaceae* and *Alcaligenaceae* induced by oral lactulose have not been previously reported. This finding is potentially significant, as their presence in the stools of patients with cirrhosis and HE has been associated with worsened inflammation/endotoxaemia and poor performance on cognitive tests<sup>15</sup>. On the contrary, decreases in *Bacteroidaceae*<sup>24,26</sup> and *Ruminococcaceae*<sup>30</sup> have been previously reported with lactulose administration. *Bacteroides* spp., are known to produce isovalerate<sup>59</sup>, as well as pro-inflammatory and barrier-disruptive neurotoxins/enterotoxins<sup>60</sup>, and  $\beta$ -glucuronidase<sup>61</sup>, a potential carcinogenic. Reduced faecal activity levels of this enzyme have been demonstrated previously with lactulose administration<sup>26,62</sup>. Besides producing butyrate<sup>63</sup>, *Ruminococcaceae* and *Lachnospiraceae* are also known to produce  $\beta$ -glucuronidase<sup>64</sup>. The lack of a significant increase in *Lactobacillaceae* with lactulose administration has been demonstrated before; hence, this study confirms that it is not a major hallmark of lactulose use<sup>24,25,29,30</sup>.

CDR is a measure of dysbiosis in humans: healthy people are reported to have a higher ratio than patients with cirrhosis, and the presence of HE was associated with an even lower CDR<sup>14</sup>. Although extrapolated from studies in people, the increase of CDR during lactulose administration could represent improvement of dysbiosis, despite an overall lower microbiota diversity and richness.

Limitations of this study include the small number of dogs, the collection of voided faecal samples rather than mucosal or luminal colonic samples, the lack of storage buffer/cryoprotectant and the fact that 16S rRNA gene sequencing allows assessment of taxonomy and abundance, but no species level resolution, nor extrapolation of functional data from the microbiome. However, given the longitudinal study design, each subject could serve as their own control, allowing observation of general trends of microbiota changes. For ethical reasons, invasive collection methods were not employed in these privately owned animals<sup>47,65</sup>. Short-term storage of faecal samples without cryopreservative or buffer is likely to not impact on major phyla distribution<sup>66,67</sup>. To investigate functional changes of the microbiome, high-throughput techniques (whole metagenome shotgun sequencing), metabolomics or metatranscriptomics could have been performed, but this was not within the scope of the current study.

In conclusion, lactulose can drive a reversible quantitative and qualitative modulation of the faecal microbiota in this dog model. Future research is warranted to focus on the investigation of microbiome dynamics in canine models of spontaneous naturally occurring HE (e.g. congenital portosystemic shunts)<sup>68</sup>. This could

include similar longitudinal studies to assess effects before and after lactulose treatment or the correlation or comparison with other management strategies. Allowing for more targeted treatment endpoints could not only advance knowledge but also improve outcomes in HE.

## Data Availability

The datasets generated and/or analysed during the current study are available in the Mendeley Data repository, <https://doi.org/10.17632/8ctyv86ccp.1>.

## References

- Bajaj, J. S. *et al.* Hepatic Encephalopathy Is Associated With Mortality in Patients With Cirrhosis Independent of Other Extrahepatic Organ Failures. *Clin Gastroenterol Hepatol.* **15**, 565–574 e564, <https://doi.org/10.1016/j.cgh.2016.09.157> (2017).
- Gadiparthi, C. *et al.* Waitlist Outcomes in Liver Transplant Candidates with High MELD and Severe Hepatic Encephalopathy. *Dig Dis Sci.* **63**, 1647–1653, <https://doi.org/10.1007/s10620-018-5032-5> (2018).
- Labenz, C. *et al.* Prospective evaluation of the impact of covert hepatic encephalopathy on quality of life and sleep in cirrhotic patients. *Aliment Pharmacol Ther.* **48**, 313–321, <https://doi.org/10.1111/apt.14824> (2018).
- Stepanova, M., Mishra, A., Venkatesan, C. & Younossi, Z. M. In-hospital mortality and economic burden associated with hepatic encephalopathy in the United States from 2005 to 2009. *Clin Gastroenterol Hepatol.* **10**, 1034–1041 e1031, <https://doi.org/10.1016/j.cgh.2012.05.016> (2012).
- Neff, G. & Zachry, W. 3rd. Systematic Review of the Economic Burden of Overt Hepatic Encephalopathy and Pharmacoeconomic Impact of Rifaximin. *Pharmacoeconomics.* **36**, 809–822, <https://doi.org/10.1007/s40273-018-0641-6> (2018).
- Ochoa-Sanchez, R. & Rose, C. F. Pathogenesis of Hepatic Encephalopathy in Chronic Liver Disease. *J Clin Exp Hepatol.* **8**, 262–271, <https://doi.org/10.1016/j.jceh.2018.08.001> (2018).
- Parekh, P. J. & Balart, L. A. Ammonia and Its Role in the Pathogenesis of Hepatic Encephalopathy. *Clin Liver Dis.* **19**, 529–537, <https://doi.org/10.1016/j.cld.2015.05.002> (2015).
- Shalimar *et al.* Prognostic Role of Ammonia in Cirrhotic Patients. *Hepatology*, <https://doi.org/10.1002/hep.30534> (2019).
- Glud, L. L., Vilstrup, H. & Morgan, M. Y. Non-absorbable disaccharides versus placebo/no intervention and lactulose versus lactitol for the prevention and treatment of hepatic encephalopathy in people with cirrhosis. *Cochrane Database Syst Rev.* CD003044, <https://doi.org/10.1002/14651858.CD003044.pub4> (2016).
- American Association for the Study of Liver, D. & European Association for the Study of the, L. Hepatic encephalopathy in chronic liver disease: 2014 practice guideline by the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases. *J Hepatol.* **61**, 642–659, <https://doi.org/10.1016/j.jhep.2014.05.042> (2014).
- Clausen, M. R. & Mortensen, P. B. Lactulose, disaccharides and colonic flora. Clinical consequences. *Drugs.* **53**, 930–942 (1997).
- Moratalla, A. *et al.* Lactulose reduces bacterial DNA translocation, which worsens neurocognitive shape in cirrhotic patients with minimal hepatic encephalopathy. *Liver Int.* **37**, 212–223, <https://doi.org/10.1111/liv.13200> (2017).
- Zhang, Z. *et al.* Large-scale survey of gut microbiota associated with MHE Via 16S rRNA-based pyrosequencing. *Am J Gastroenterol.* **108**, 1601–1611, <https://doi.org/10.1038/ajg.2013.221> (2013).
- Bajaj, J. S. *et al.* Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J Hepatol.* **60**, 940–947, <https://doi.org/10.1016/j.jhep.2013.12.019> (2014).
- Bajaj, J. S. *et al.* Linkage of gut microbiome with cognition in hepatic encephalopathy. *Am J Physiol Gastrointest Liver Physiol.* **302**, G168–175, <https://doi.org/10.1152/ajpgi.00190.2011> (2012).
- Iebba, V. *et al.* Combining amplicon sequencing and metabolomics in cirrhotic patients highlights distinctive microbiota features involved in bacterial translocation, systemic inflammation and hepatic encephalopathy. *Sci Rep.* **8**, 8210, <https://doi.org/10.1038/s41598-018-26509-y> (2018).
- Bajaj, J. S. *et al.* Randomised clinical trial: Lactobacillus GG modulates gut microbiome, metabolome and endotoxemia in patients with cirrhosis. *Aliment Pharmacol Ther.* **39**, 1113–1125, <https://doi.org/10.1111/apt.12695> (2014).
- Bajaj, J. S. *et al.* Diet affects gut microbiota and modulates hospitalization risk differentially in an international cirrhosis cohort. *Hepatology.* **68**, 234–247, <https://doi.org/10.1002/hep.29791> (2018).
- Bajaj, J. S. *et al.* Fecal microbiota transplant from a rational stool donor improves hepatic encephalopathy: A randomized clinical trial. *Hepatology.* **66**, 1727–1738, <https://doi.org/10.1002/hep.29306> (2017).
- Ziada, D. H., Soliman, H. H., El Yamany, S. A., Hamisa, M. F. & Hasan, A. M. Can Lactobacillus acidophilus improve minimal hepatic encephalopathy? A neurometabolite study using magnetic resonance spectroscopy. *Arab J Gastroenterol.* **14**, 116–122, <https://doi.org/10.1016/j.ajg.2013.08.002> (2013).
- Riggio, O. *et al.* Effect of lactitol and lactulose administration on the fecal flora in cirrhotic patients. *J Clin Gastroenterol.* **12**, 433–436 (1990).
- Sarangi, A. N., Goel, A., Singh, A., Sasi, A. & Aggarwal, R. Faecal bacterial microbiota in patients with cirrhosis and the effect of lactulose administration. *BMC Gastroenterol.* **17**, 125, <https://doi.org/10.1186/s12876-017-0683-9> (2017).
- Bajaj, J. S. *et al.* A longitudinal systems biology analysis of lactulose withdrawal in hepatic encephalopathy. *Metab Brain Dis.* **27**, 205–215, <https://doi.org/10.1007/s11011-012-9303-0> (2012).
- Terada, A., Hara, H., Kataoka, M. & Mitsuoka, T. Effect of Lactulose on the Composition and Metabolic Activity of the Human Faecal Flora. *Microbial Ecology in Health and Disease.* **5**, 43–50, <https://doi.org/10.3109/08910609209141303> (1992).
- Bouhnik, Y. *et al.* Lactulose ingestion increases faecal bifidobacterial counts: a randomised double-blind study in healthy humans. *Eur J Clin Nutr.* **58**, 462–466, <https://doi.org/10.1038/sj.ejcn.1601829> (2004).
- Ballongue, J., Schumann, C. & Quignon, P. Effects of lactulose and lactitol on colonic microflora and enzymatic activity. *Scand J Gastroenterol Suppl.* **222**, 41–44, <https://doi.org/10.1080/00365521.1997.11720716> (1997).
- Tuohy, K. M. *et al.* A Human Volunteer Study to Determine the Prebiotic Effects of Lactulose Powder on Human Colonic Microbiota. *Microbial Ecology in Health and Disease.* **14**, 165–173, <https://doi.org/10.1080/089106002320644357> (2002).
- De Preter, V. *et al.* Effect of lactulose and Saccharomyces boulardii administration on the colonic urea-nitrogen metabolism and the bifidobacteria concentration in healthy human subjects. *Aliment Pharmacol Ther.* **23**, 963–974, <https://doi.org/10.1111/j.1365-2036.2006.02834.x> (2006).
- Vanhoutte, T. *et al.* Molecular monitoring of the fecal microbiota of healthy human subjects during administration of lactulose and Saccharomyces boulardii. *Appl Environ Microbiol.* **72**, 5990–5997, <https://doi.org/10.1128/AEM.00233-06> (2006).
- Venema, K., van Nuenen, M. H. M. C., van den Heuvel, E. G., Pool, W. & van der Vossen, J. M. B. M. The Effect of Lactulose on the Composition of the Intestinal Microbiota and Short-chain Fatty Acid Production in Human Volunteers and a Computer-controlled Model of the Proximal Large Intestine. *Microbial Ecology in Health and Disease.* **15**, 94–105, <https://doi.org/10.1080/08910600310019895> (2003).
- Dong, T. S. & Gupta, A. Influence of Early Life, Diet, and the Environment on the Microbiome. *Clin Gastroenterol Hepatol*, <https://doi.org/10.1016/j.cgh.2018.08.067> (2018).
- Coelho, L. P. *et al.* Similarity of the dog and human gut microbiomes in gene content and response to diet. *Microbiome.* **6**, 72, <https://doi.org/10.1186/s40168-018-0450-3> (2018).

33. Axelsson, E. *et al.* The genomic signature of dog domestication reveals adaptation to a starch-rich diet. *Nature*. **495**, 360–364, <https://doi.org/10.1038/nature11837> (2013).
34. Lidbury, J. A., Cook, A. K. & Steiner, J. M. Hepatic encephalopathy in dogs and cats. *J Vet Emerg Crit Care (San Antonio)*. **26**, 471–487, <https://doi.org/10.1111/vec.12473> (2016).
35. PowerSoil<sup>®</sup> DNA Isolation Kit Instruction Manual, *MO BIO Laboratories, Inc.*, <https://www.qiagen.com/us/resources/download.aspx?id=5c00f8e4-c9f5-4544-94fa-653a5b2a6373&lang=en> (2014).
36. Caporaso, J. G. *et al.* Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* **108**(Suppl 1), 4516–4522, <https://doi.org/10.1073/pnas.1000080107> (2011).
37. Caporaso, J. G. *et al.* Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* **6**, 1621–1624, <https://doi.org/10.1038/ismej.2012.8> (2012).
38. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. **13**, 581–583, <https://doi.org/10.1038/nmeth.3869> (2016).
39. Coordinators, N. R. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* **46**, D8–D13, <https://doi.org/10.1093/nar/gkx1095> (2018).
40. Katoh, K. & Standley, D. M. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol.* **30**, 772–780, <https://doi.org/10.1093/molbev/mst010> (2013).
41. Lane, D. In *Nucleic Acid Techniques in Bacterial Systematics* (eds Stackebrandt, E. & Goodfellow, M.) 115–175 (John Wiley and Sons, 1991).
42. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS One*. **5**, e9490, <https://doi.org/10.1371/journal.pone.0009490> (2010).
43. Bokulich, N. A. *et al.* Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*. **6**, 90, <https://doi.org/10.1186/s40168-018-0470-z> (2018).
44. Bajaj, J. S. *et al.* Association Between Intestinal Microbiota Collected at Hospital Admission and Outcomes of Patients with Cirrhosis. *Clin Gastroenterol Hepatol.* <https://doi.org/10.1016/j.cgh.2018.07.022> (2018).
45. Anderson, M. J. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*. **26**, 32–46, <https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x> (2001).
46. Zhai, S., Zhu, L., Qin, S. & Li, L. Effect of lactulose intervention on gut microbiota and short chain fatty acid composition of C57BL/6J mice. *Microbiologyopen*. e00612, <https://doi.org/10.1002/mbo3.612> (2018).
47. Mao, B. *et al.* Lactulose Differently Modulates the Composition of Luminal and Mucosal Microbiota in C57BL/6J Mice. *J Agric Food Chem.* **64**, 6240–6247, <https://doi.org/10.1021/acs.jafc.6b02305> (2016).
48. Chae, J. P., Pajarillo, E. A. B., Park, C. S. & Kang, D. K. Lactulose increases bacterial diversity and modulates the swine faecal microbiome as revealed by 454-pyrosequencing. *Anim Feed Sci Tech.* **209**, 157–166, <https://doi.org/10.1016/j.anifeedsci.2015.07.018> (2015).
49. Chae, J. P., Pajarillo, E. A., Oh, J. K., Kim, H. & Kang, D. K. Revealing the combined effects of lactulose and probiotic enterococci on the swine faecal microbiota using 454 pyrosequencing. *Microb Biotechnol.* **9**, 486–495, <https://doi.org/10.1111/1751-7915.12370> (2016).
50. Herstad, K. M. V. *et al.* A diet change from dry food to beef induces reversible changes on the faecal microbiota in healthy, adult client-owned dogs. *BMC Vet Res.* **13**, 147, <https://doi.org/10.1186/s12917-017-1073-9> (2017).
51. Kim, J., An, J. U., Kim, W., Lee, S. & Cho, S. Differences in the gut microbiota of dogs (*Canis lupus familiaris*) fed a natural diet or a commercial feed revealed by the Illumina MiSeq platform. *Gut Pathog.* **9**, 68, <https://doi.org/10.1186/s13099-017-0218-5> (2017).
52. Middleton, R. P. *et al.* Metabolic Differences between Dogs of Different Body Sizes. *J Nutr Metab.* **2017**, 4535710, <https://doi.org/10.1155/2017/4535710> (2017).
53. Hand, D., Wallis, C., Colyer, A. & Penn, C. W. Pyrosequencing the canine faecal microbiota: breadth and depth of biodiversity. *PLoS One*. **8**, e53115, <https://doi.org/10.1371/journal.pone.0053115> (2013).
54. Kolenbrander, P. The Genus *Veillonella*. *Prokaryotes: A Handbook on the Biology of Bacteria, Vol 4, Third Edition*, 1022–1040, [https://doi.org/10.1007/0-387-30744-3\\_36](https://doi.org/10.1007/0-387-30744-3_36) (2006).
55. Sandri, M., Dal Monego, S., Conte, G., Sgorlon, S. & Stefanon, B. Raw meat based diet influences faecal microbiome and end products of fermentation in healthy dogs. *Bmc Veterinary Research.* **13**, <https://doi.org/10.1186/s12917-017-0981-z> (2017).
56. Bothe, M. K. *et al.* Dose-Dependent Prebiotic Effect of Lactulose in a Computer-Controlled *In Vitro* Model of the Human Large Intestine. *Nutrients.* **9**, <https://doi.org/10.3390/nu9070767> (2017).
57. Pokusaeva, K., Fitzgerald, G. F. & van Sinderen, D. Carbohydrate metabolism in *Bifidobacteria*. *Genes Nutr.* **6**, 285–306, <https://doi.org/10.1007/s12263-010-0206-6> (2011).
58. Belenguer, A. *et al.* Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. *Appl Environ Microbiol.* **72**, 3593–3599, <https://doi.org/10.1128/AEM.72.5.3593-3599.2006> (2006).
59. Ito, Y. *et al.* Effect of lactulose on short-chain fatty acids and lactate production and on the growth of faecal flora, with special reference to *Clostridium difficile*. *J Med Microbiol.* **46**, 80–84, <https://doi.org/10.1099/00222615-46-1-80> (1997).
60. Lukiw, W. J. The microbiome, microbial-generated proinflammatory neurotoxins, and Alzheimer's disease. *J Sport Health Sci.* **5**, 393–396, <https://doi.org/10.1016/j.jsbs.2016.08.008> (2016).
61. Wallace, B. D. *et al.* Structure and Inhibition of Microbiome beta-Glucuronidases Essential to the Alleviation of Cancer Drug Toxicity. *Chem Biol.* **22**, 1238–1249, <https://doi.org/10.1016/j.chembiol.2015.08.005> (2015).
62. De Preter, V. *et al.* Effect of dietary intervention with different pre- and probiotics on intestinal bacterial enzyme activities. *Eur J Clin Nutr.* **62**, 225–231, <https://doi.org/10.1038/sj.ejcn.1602706> (2008).
63. Louis, P. & Flint, H. J. Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol.* **19**, 29–41, <https://doi.org/10.1111/1462-2920.13589> (2017).
64. Gloux, K. *et al.* A metagenomic beta-glucuronidase uncovers a core adaptive function of the human intestinal microbiome. *Proc Natl Acad Sci USA* **108**(Suppl 1), 4539–4546, <https://doi.org/10.1073/pnas.1000066107> (2011).
65. Bajaj, J. S. *et al.* Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. *Am J Physiol Gastrointest Liver Physiol.* **303**, G675–G685, <https://doi.org/10.1152/ajpgi.00152.2012> (2012).
66. Horng, K. R., Ganz, H. H., Eisen, J. A. & Marks, S. L. Effects of preservation method on canine (*Canis lupus familiaris*) fecal microbiota. *PeerJ.* **6**, e4827, <https://doi.org/10.7717/peerj.4827> (2018).
67. Song, S. J. *et al.* Preservation Methods Differ in Fecal Microbiome Stability, Affecting Suitability for Field Studies. *mSystems.* **1**, <https://doi.org/10.1128/mSystems.00021-16> (2016).
68. Tivers, M. S. *et al.* Hyperammonemia and systemic inflammatory response syndrome predicts presence of hepatic encephalopathy in dogs with congenital portosystemic shunts. *PLoS One.* **9**, e82303, <https://doi.org/10.1371/journal.pone.0082303> (2014).

## Acknowledgements

The authors would like to thank Edinburgh Genomics, The University of Edinburgh, for performing the DNA sequencing and generating the raw data. Edinburgh Genomics is partly supported through core grants from NERC (R8/H10/56), MRC (MR/K001744/1) and BBSRC (BB/J004243/1). The authors would also like to thank the owners who consented their dogs to participate in this study. This study was supported by a *Royal (Dick) School of Veterinary Studies Clinical Research Grant*. The funding source had no involvement in: the study design; the collection, analysis and interpretation of data; the writing of the manuscript; or the decision to submit the manuscript for publication. J.J.S. is a University of Edinburgh Chancellor's Fellow based at the Roslin Institute. He is supported by strategic funding from the Biotechnology and Biosciences Research Council (BB/P013759/1 and BB/P013732/1). R.J.M. and M.S. were supported by BBSRC through the Institute Strategic Programme funding (BB/J004235/1 and BB/P013740/1).

## Author Contributions

J.J.S., R.J.M. and A.G.G. developed study concept and design. M.F.F., J.J.S., D.N.C., S.M.C. and D.E.G. acquired data. M.F.F., S.S.S. and M.S. analysed and interpreted data. M.F.F. and M.S. performed statistical analysis. M.F.F. drafted the manuscript. All authors reviewed the manuscript. J.J.S., D.N.C., S.M.C., D.E.G., R.J.M. and A.G.G. provided administrative, technical and/or material support. S.S.S., J.J.S., R.J.M., A.G.G. and M.S. undertook study supervision.

## Additional Information

**Competing Interests:** The authors declare no competing interests.

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019